Identification of a role for *Saccharomyces cerevisiae* Cgr1p in pre-rRNA processing and 60S ribosome subunit synthesis

Terence I. Moy, Douglas Boettner, Judith C. Rhodes, Pamela A. Silver and David S. Askew

Author for correspondence: David S. Askew. Tel: +1 513 558 2395. Fax: +1 513 558 2141. e-mail: David.Askew@uc.edu

*Saccharomyces cerevisiae* CGR1 encodes a conserved fungal protein that localizes to the nucleolus. To determine if this localization reflects a role for Cgr1p in ribosome biogenesis two yeast cgr1 mutants were examined for defects in ribosome synthesis: a conditional depletion strain in which *CGR1* is under the control of a tetracycline-repressible promoter and a mutant strain in which a C-terminal truncated Cgr1p is expressed. Both strains had impaired growth rates and were hypersensitive to the aminoglycosides paromomycin and hygromycin. Polysome analyses of the mutants revealed increased levels of free 40S subunits relative to 60S subunits, a decrease in 80S monosomes and accumulation of half-mer polysomes. Pulse–chase labelling demonstrated that pre-rRNA processing was defective in the mutants, resulting in accumulation of the 35S, 27S and 7S pre-rRNAs and delayed production of the mature 25S and 5S rRNAs. The synthesis of the 18S and 5S rRNAs was unaffected. Loss of Cgr1 function also caused a partial delocalization of the 5′-ITS1 RNA and the nucleolar protein Nop1p into the nucleoplasm, suggesting that Cgr1p contributes to compartmentalization of nucleolar constituents. Together these findings establish a role for Cgr1p in ribosome biogenesis.

**Keywords:** yeast, nucleolus, ribosome biogenesis

INTRODUCTION

Ribosome synthesis plays an essential role in the cellular adaptation to nutrient availability in all micro-organisms, allowing for the tight coordination of protein synthetic capacity with the demand for rapid growth (reviewed in Planta, 1997; Warner, 1999; Nomura, 1999). In eukaryotic organisms, ribosome synthesis takes place primarily in the nucleolus, a specialized compartment of the nucleus that is responsible for the transcription of the rDNA genes, the processing of pre-rRNA and the coordinated assembly of pre-rRNAs with ribosomal proteins (reviewed by Scher & Weisenberger, 1994; Shaw & Jordan, 1995; Venema & Tollervey, 1999). Most of our understanding of eukaryotic ribosome synthesis has been obtained from the model yeast *Saccharomyces cerevisiae* (reviewed by Planta, 1997; Warner, 1999; Nomura, 1999). The yeast rDNA is transcribed by RNA polymerase I as a single 35S precursor RNA that is processed into three mature rRNAs (reviewed by Warner, 1982; Raué & Planta, 1991; Venema & Tollervey, 1999). The 35S pre-rRNA is flanked by two external transcribed spacers (ETSs), 5′-ETS and 3′-ETS, and contains two internal transcribed spacers (ITSs), ITS1 and ITS2, that separate the sequences for the three mature rRNAs: 18S, 5′-ETS and 25S (see Fig. 1). The mature 5S and 25S rRNAs become incorporated into the 60S ribosome, and the mature 18S rRNA becomes part of the 40S ribosome. A fourth rRNA (5S) is transcribed independently by RNA polymerase III from a separate transcription unit and this RNA is incorporated into the 60S ribosome. The removal of the spacer sequences from the pre-rRNAs, and the final maturation of the rRNAs, is a complex process that involves a number of non-ribosomal proteins and small nucleolar RNAs (Raué & Planta, 1991; Kressler *et al*., 1999).

Since ribosome synthesis is tightly linked to growth, the ability to target nucleolar proteins involved in ribosome biogenesis would be an effective therapeutic strategy against fungal infections. However, the ability to design

**Abbreviations:** ETS, external transcribed spacer; ITS, internal transcribed spacer.
selective inhibitors of fungal ribosome synthesis is limited by insufficient knowledge of the proteins involved in ribosome biogenesis in pathogenic fungi. We have previously identified a yeast nucleolar protein, Cgr1p, that is highly conserved among fungi. Species homologues have been cloned from Cgr1p, that is highly conserved among fungi. Species have previously identified a yeast nucleolar protein, involved in ribosome biogenesis in pathogenic fungi. We limited by insufficient knowledge of the proteins in-selective inhibitors of fungal ribosome synthesis is 

Fig. 1. The major pre-rRNA processing pathways in S. cerevisiae. The 35S pre-rRNA is transcribed in the nucleolus and processed into the mature 185, 5′-8S and 25S rRNAs by a series of endonucleolytic and exonucleolytic steps. The 35S pre-rRNA is cleaved at site A0 to generate the 35S pre-rRNA and site A1, to generate the 325 pre-rRNA. The 325 pre-rRNA is then cleaved at A2 to produce the 205 and 275A2 pre-rRNAs, thereby separating the RNAs destined for the small and large ribosomal subunits. Final maturation of the 205 precursor into the 185 rRNA occurs in the cytoplasm. The 275A2 precursor is processed by one of two alternative pathways into the mature 5′-8S and 25S rRNAs. These pathways are distinguished by the synthesis of either a long or short version of the 5′-8S rRNA (the long version is indicated by the black box). The 275 precursors of both pathways are eventually processed at sites C1 and C2 to yield the 7S pre-rRNA and the mature 25S rRNA. Subsequent 3′→5′ exonucleolytic digestion of the 7S rRNA generates the mature 5′-8S rRNA. Figure modified with permission from Hong et al. (1997).

METHODS

Strains and culture conditions. The yeast strains used in this study are listed in Table 1. Yeasts were grown on either rich medium [YPD: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose], synthetic medium [SD: 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) dextrose] that was supplemented to meet auxotrophic requirements for each strain, or synthetic drop-out medium (Burke et al., 2000). Generation times were calculated from cultures that were maintained in exponential growth for at least 30 h by diluting whenever the OD600 reached 0.7. To measure antibiotic sensitivity, 1 × 10⁷ exponentially growing cells were plated in a top agar overlay [1% (w/v) agarose in YPD] onto YPD plates. A 0.75 cm diameter glass filter disc was then placed on the surface of each plate (Brandel) and 10 µl of a dilution of either paromomycin (Sigma) or hygromycin (Sigma) was spotted onto each disc. The plates were cultured for 4 days at 30°C and the diameter of the zone of growth inhibition around each filter was used as a measure of drug sensitivity.

Plasmids. A tetracycline-repressible CGR1 expression plasmid was constructed by amplifying the CGR1 ORF from yeast genomic DNA (strain YPH50) using the forward primer 5′-CGATGGATCCAGGAATATGGAATGAAACAGG-AG-3′ and the reverse primer 5′-CGATATCGATCTAACCAGTTCTTTTGACC-3′ (restriction sites are in italic). The PCR product was cloned into the pCM183 plasmid (Gari et al., 1997) at the BamHI and ClaI sites of the poly linker to make pDA361 (Table 1). This construction placed the CGR1 ORF downstream of a hybrid promoter comprising two copies of the tet operator sequence linked to the CYC1 promoter, and upstream of the CYC1 terminator.

Gel electrophoresis and blotting. For analysis of gene expression, RNA was isolated from yeast cultures by disruption with 0.5 mm glass beads (Fisher Scientific) according
Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype/construction</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry1(Y300)</td>
<td>MATα len2-3,112 trp1-1 ura3-1 his3-11,15 can1-100</td>
<td>Y. Sanchez, University of Cincinnati</td>
</tr>
<tr>
<td>YDA110</td>
<td>MATα len2-3,112 trp1-1 ura3-1 his3-11,15 can1-100 cgr1Δ81-120::kan'</td>
<td>This study</td>
</tr>
<tr>
<td>YDA128</td>
<td>MATα len2-3,112 trp1-1 ura3-1 his3-11,15 can1-100 cgr1α::kan'(pDA361)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCM183</td>
<td>P&lt;sub&gt;total&lt;/sub&gt;CYC1 TRP1 CEN</td>
<td>ATCC</td>
</tr>
<tr>
<td>pDA361</td>
<td>pCM183, expressing S. cerevisiae CGR1</td>
<td>This study</td>
</tr>
</tbody>
</table>

to standard procedures. Twenty micrograms of total RNA was fractionated by formaldehyde gel electrophoresis as previously described (Li et al., 1997), transferred to positively charged nylon membranes (MSI) and hybridized to <sup>32</sup>P-labelled DNA probes under stringent conditions in 50% (v/v) formamide/5 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.6, 2 x Denhardt’s solution, 10% (w/v) dextran sulfate, 1% (w/v) SDS). Hybridization intensity was quantified with a Phosphorimager (Molecular Dynamics) and normalized for loading by quantitating the relative levels of SYBR green (Molecular Probes) stained 18S rRNA on a Phosphorimager.

Genomic DNA was extracted by cell disruption in the presence of 0.5 mm diameter glass beads (Fisher Scientific) according to standard protocols. For genomic Southern blot analysis, 10 µg digested DNA was fractionated on a 1% (w/v) agarose gel, transferred to a nylon membrane (MSI) and hybridized to a <sup>32</sup>P<sub>dCTP</sub>-labelled probe under conditions of high stringency as previously described (Li et al., 1997). Hybridization was monitored using a Phosphorimager (Molecular Dynamics).

**Construction of a cgr1 truncation mutant and a cgr1 deletion mutant.** A mutant expressing a C-terminal truncation of Cgr1p, YDA110, was constructed by one-step PCR-mediated gene disruption. The truncation cassette was obtained by PCR amplification of the kanamycin-resistance gene (kan') using plasmid pFA6-kanMX2 (Wach et al., 1994) as the template and primers containing 50 bp CGR1-homologous sequences. The upstream primer was 5'-AGCTCGTTTAAAC«AACCATGTTAAAGTGAGGCGCGCCACTTCTAA-3' and the downstream primer was 5'-TATGAGCCTCTCATATGCTTTATACCATTGTGCTTATCCGAATTCG-3' (sequences in italic are specific to pFA6-3HA-kanMX6). This construction places a stop codon and the downstream primer was 5'-CTGTCTAGGGATGCCCT-TATTTTCTCCTTGTCAGCTTAATAATGTGCTAG-GCCACTAGTTGGATCTG-3' (sequences in italic are specific to pFA6-3HA-kanMX2). Following selection on G418 plates, the colonies were genotyped by PCR and deletion of the CGR1 ORF was confirmed by genomic Southern blot analysis, using radiolabelled probes specific for the CGR1 and kan' genes. Down-regulation of the tetO-CYC1 promoter in pDA361 was accomplished by supplementing the medium with 2 µg ml<sup>-1</sup> of the tetracycline analogue doxycycline, as described by Gari et al. (1997).

**Pulse–chase analysis of rRNA processing.** For the [<sup>3</sup>H]methionine pulse–chase experiments, wild-type and YDA110 cells were grown to a density of (1–2) × 10<sup>7</sup> cells ml<sup>-1</sup> in methionine-free media at 25 °C. Wild-type cells containing pCM183 (Table 1) and YDA128 were grown to a density of (2–4) × 10<sup>7</sup> cells ml<sup>-1</sup> in methionine-free media at 25 °C. The cells were diluted into media containing 2 µg doxycycline ml<sup>-1</sup> and cultured for 29 h. A total of 10<sup>8</sup> cells was concentrated to a volume of 1 ml and pulse labelled with 250 µCi (9.25 MBq) [<sup>3</sup>H]methionine for 2 min (70–85 Ci mmol<sup>-1</sup>, 5 mCi ml<sup>-1</sup>, 185 MBq ml<sup>-1</sup>; Amersham-Pharmacia Biotech). For the zero time point, 250 µl cells was transferred to a new tube, washed in 1 ml ice-cold media and the cell pellet frozen on dry ice. Chase was initiated by adding unlabelled methionine to the culture at a concentration of 1 mg methionine ml<sup>-1</sup>. At time points of 2, 5 and 10 min of chase, 250 µl cells were removed and processed as described above. RNA was isolated by the hot acid phenol method (Ausubel et al., 1997). A total of 20000 c.p.m. radioactivity was loaded per lane onto a 12% (w/v) agarose-formaldehyde gel. RNA was transferred to a nylon membrane (Hybond-N+, Amersham-Pharmacia), UV-cross-linked and sprayed with En'hance (NEN). The membrane was exposed to film for 1 day at −80 °C.

For [<sup>5</sup>,<sup>6</sup>-<sup>3</sup>H]uracil pulse–chase experiments, wild-type and YDA110 cells containing pRS306 (URA3 CEN) were grown to a density of 1 × 10<sup>7</sup> cells ml<sup>-1</sup> in media lacking uracil at 25 °C. A total of 5 × 10<sup>8</sup> cells was concentrated to a volume of 6 ml
and labelled with 100 µCi (37 MBq) [5,6-3H]uracil (30–50 Ci mmol−1, 1 mCi ml−1, 37 MBq ml−1; NEN). After labeling for 3 and 6 min, 1 ml culture was removed, centrifuged, supernatants removed, and cell pellets frozen. Chase was initiated by adding unlabelled uracil to a concentration of 20 µg uracil ml−1. At time points of 5, 10, 30 and 60 min of chase (for the 6 min label), a 1 ml sample was processed as described above and 5 µg RNA from each time point was loaded onto a 8% polyacrylamide, 8 M urea gel. RNA was transferred to Hybond-N+ membranes by semi-dry electrophoresis (Owl Scientific) and visualized by fluorography as described above. The membrane was exposed to film for 15 days at −80°C.

**Polysome profile analysis.** Polysome profiles were performed as described by Kressler et al. (1997) with the following modifications. Cells were taken from the cultures used for the [methyl-3H]methionine pulse–chase experiments described above, and the lysate (4 A260 units) was layered onto 10 ml linear 7–49% sucrose gradients. Samples were centrifuged in a Beckman SW41Ti rotor for 2 h at 39000 r.p.m. at 4°C (Beckman Instruments). A Beckman fraction recovery system was used to pass the gradients through a Pharmacia UV-1 monitor to measure A260. Analysis of dissociated ribosomal subunits was performed as described by Kressler et al. (1997).

**Subcellular localization of Nop1p and 5′-ITS1.** Nop1p was visualized with anti-Nop1p monoclonal antibodies as previously described (Moy & Silver 1999). 5′-ITS1 rRNA was visualized by fluorescence in situ hybridization as described by Amberg et al. (1992) with the following modifications. An oligonucleotide complementary to the first 50 bases of ITS1 rRNA, 5′-ATGCTTGGCTAAAAACAAAAAATCCATTTC-AAAATTATTATTCTT-3′, was synthesized with the Cy3 fluorophore at its 5′ end (IDT). Samples were hybridized with this oligonucleotide at a concentration of 50 nM.

**RESULTS**

**Mutants of CGR1 are growth impaired.**

We have previously reported that CGR1 is essential in the YPH274 background (Sun et al., 2001a). To date, we have microdissected a total of 64 tetrads from a CGR1/cgr1A heterozygote constructed in the YPH274 strain, each indicating that spore viability segregates with the wild-type allele. A slow-growing cgr1A mutant has recently been reported using meiotic segregation analysis by the yeast deletion project (Winzeler et al., 1999), suggesting that Cgr1p may be non-essential in some strains. As a first step to determine if Cgr1p has a role in ribosome synthesis in S. cerevisiae, we constructed two haploid mutants that were deficient in Cgr1p function: a conditional Cgr1p deletion mutant and a C-terminal cgr1 truncation mutant. The deletion strain, YDA128, was created by deleting the chromosomal CGR1 ORF in a haploid strain that contained a plasmid-borne CGR1 gene under the control of a tacrycline-repressible promoter (see Methods). Genomic Southern blot analysis of YDA128 showed homologous targeting of the CGR1 allele (data not shown). Northern blot analysis was used to confirm that YDA128 cells treated with doxycycline were expressing levels of CGR1 that were below the levels seen in wild-type cells. In the absence of doxycycline, exponential cultures of YDA128 expressed 17-fold more CGR1 RNA than wild-type cells (Fig. 2, compare wild-type with the zero time point for YDA128). However, within 6 h of treatment with doxycycline, CGR1 expression was repressed (Fig. 2), falling over 10-fold below the levels observed in exponentially growing wild-type cells. When overnight cultures of wild-type and YDA128 cells were diluted into fresh medium containing doxycycline, and maintained in exponential phase by diluting whenever the culture reached an OD600 of 0.7, the cultures initially grew with the same generation time of 2.3 h. However, after 12–15 h in doxycycline, the generation time of YDA128 was increased to 5.5 h (Fig. 3). Doxycycline had no effect on the growth rate of wild-type cells at the concentrations used in this experiment (data not shown). Since extended exposure of the Northern blot shown in Fig. 2 showed that some CGR1 expression remained in the doxycycline-treated YDA128 cells, this promoter was not completely repressed in the presence of doxycycline. However, the results indicate that wild-type levels of CGR1 mRNA are required for optimal growth, thus providing an opportunity to determine how reduced CGR1 mRNA affects ribosome synthesis.

A second mutant strain, YDA110, was constructed by introducing a stop codon into the chromosomal CGR1 gene, thereby truncating the expressed protein at amino acid 81. Homologous targeting of the CGR1 allele was confirmed by genomic Southern blot analysis (data not shown). This mutant was also growth impaired (generation time 3.8 h), but to a lesser extent than doxycycline-treated YDA128 cells (generation time 5.5 h), suggesting that the truncated protein has residual activity. These results indicate that a truncated Cgr1p is
Cgr1p deficiency impairs growth. Overnight cultures of wild-type and YDA128 cells were seeded into YPD medium containing 2 µg doxycycline ml⁻¹ and diluted whenever the culture attained an OD₆₀₀ of 0.7 to maintain exponential growth. Wild-type: cry1 cells containing the empty pCM183 plasmid (Table 1).

Fig. 3. Cgr1p deficiency impairs growth. Cells expressing a truncated Cgr1p show increased sensitivity to translational inhibitors. Filter paper circles containing 10 µl of an antibiotic concentration were placed onto a YPD plate containing 1 × 10⁸ wild-type (a and c) or YDA110 (b and d) cells. (a) and (b) contain hygromycin (top left, 0 mg ml⁻¹; top right, 50 mg ml⁻¹; bottom left, 100 mg ml⁻¹; bottom right, 250 mg ml⁻¹). (c) and (d) contain paromomycin (top left, 0 mg ml⁻¹; top right, 50 mg ml⁻¹; bottom right, 200 mg ml⁻¹; bottom left, 500 mg ml⁻¹). Wild-type, Cry1.

cgr1 mutants are hypersensitive to translation inhibitors

Yeast mutants deficient in ribosome assembly or function often display increased sensitivity to aminoglycoside antibiotics (Zanchin et al., 1997; Benard et al., 1998; Ho & Johnson, 1999; Dresios et al., 2000). We have previously shown that a strain expressing CGR1 from a glucose-repressible promoter is hypersensitive to the aminoglycoside paromomycin under repressing conditions (Sun et al., 2001a). To determine whether the cgr1 truncation mutant also confers increased susceptibility to translational inhibitors, the sensitivity of YDA110 cells to paromomycin and hygromycin was examined. Glass filter discs containing various concentrations of each antibiotic were placed onto the surface of a plate of confluent cells and cultured for 4 days at 30 °C. As shown by the zone of growth inhibition surrounding the filter paper discs, strain YDA110 was more sensitive to both of these antibiotics than wild-type cells (Fig. 4), indicating that inhibitors of translational fidelity synergize with loss of Cgr1 function to inhibit growth.

cgr1 mutants are deficient in the synthesis of 60S ribosomal subunits

To determine if Cgr1p has a role in ribosome synthesis, the polysome profile of each cgr1 mutant was analysed on sucrose density gradients and compared to that of wild-type cells. As shown in Fig. 5, wild-type cells showed the typical profile of 40S and 60S subunits, the 80S monosome, and polysome peaks representing 2–8 ribosomes. By contrast, YDA110 cells showed a reduction in the level of the 60S subunit relative to free 40S subunits, a reduction in the level of the 80S monosome peak, an overall decrease in the mean number of ribosomes in polysomes, and the appearance of half-mer polysomes (Fig. 5). Half-mer polysomes, evident as insufficient to support optimal growth, thus providing an independent mutant in which to assess the consequences of impaired Cgr1p function on ribosome synthesis.
small peaks following the 80S and first two polysome peaks, represent 43S pre-initiation complexes composed of mRNA, the 40S subunit and initiation factors (Helser et al., 1981). The reduction in the level of the 60S subunit, and presence of half-mers, suggested a stoichiometric imbalance between 60S and 40S subunits. This was confirmed by examining the relative amounts of total 60S and 40S subunits under conditions that cause dissociation of the subunits. A normal ratio of 60S:40S subunits of around 2 was observed for wild-type cells. By contrast, the ratio of 60S:40S subunits in YDA110 cells was approximately equivalent, confirming a disruption in 60S:40S stoichiometry (data not shown). YDA128 cells depleted of Cgr1p by growth in doxycycline for 24 h showed the same polysome profile as YDA110 cells (data not shown), indicating that either Cgr1p truncation, or a decrease in the levels of the wild-type protein, adversely affects the synthesis of the 60S subunit.

**Cgr1p is required for normal pre-rRNA processing**

The polysome profiles of the cgr1 mutant shown in Fig. 5 indicated that Cgr1p is required for the maintenance of normal 60S ribosomal subunit levels. Since a decrease in the level of a ribosomal subunit is often caused by a defect in pre-rRNA processing and/or assembly of ribosomal subunits (Kressler et al., 1999; Venema & Tollervey, 1999), we examined the synthesis and processing of pre-rRNA in each of the mutants. The major pre-rRNA processing pathways in *S. cerevisiae* are depicted in Fig. 1. Since the 18S and 25S rRNAs are highly methylated, their synthesis and processing can be readily followed by metabolic labelling with [methyl-3H]methionine (Warner, 1991). Exponentially growing cultures in medium lacking methionine were pulse-labelled for 2 min with [methyl-3H]methionine and chased for 2, 5 and 10 min with an excess of cold methionine. Total RNA was extracted from the cells, fractionated by formaldehyde-agarose gel electrophoresis and analysed for the presence of pre-rRNA species as described in Methods. As expected, the wild-type 35S and 32S precursors disappeared within 2 min of the chase (Fig. 6a), indicating normal and rapid processing to the 27S and 20S intermediates. After 5 min chasing, the majority of the label was present in the mature 25S and 18S rRNAs, and by 10 min only the mature rRNAs were evident (Fig. 6a). By contrast, the 27S pre-rRNA accumulated in the YDA110 strain and only a small amount of this intermediate was processed into the 25S rRNA, even after 10 min chasing (Fig. 6a). Consistent with this delay in 27S pre-rRNA processing was a slight reduction in the steady-state levels of the mature 25S rRNA (data not shown). The processing of the 35S pre-rRNA was also slightly delayed, but since there was no accumulation of the 20S pre-rRNA, and the appearance of the 18S rRNA occurred with the same kinetics as wild-type, we conclude that the truncated Cgr1 protein was interfering with processing of the 27S rRNA.

To determine if the effect on 25S rRNA synthesis was due to a general loss of Cgr1 function or to an abnormal effect of the truncated protein, the same experiments were performed on cells depleted of Cgr1p. Exponential cultures of YDA128 grown in the presence of doxycycline for 24 h were pulse–chase labelled with [methyl-3H]methionine and analysed for processing of the 35S pre-rRNA as described above. As shown in Fig. 6(b), wild-type cells containing the empty tetracycline-repressible plasmid processed the 35S pre-rRNA with the same kinetics as wild-type cells, indicating that doxycycline treatment did not adversely affect pre-rRNA processing. By contrast, YDA128 cells depleted of Cgr1p showed the same defect in the processing of the 27S pre-rRNA as YDA110 cells, with normal processing of the 20S pre-rRNA (Fig. 6b). A small amount of the 23S rRNA may be increased in YDA110 and YDA128 after 5 min chasing (Fig. 6a, b). The 23S pre-rRNA is an unusual processing intermediate leading to the production of the 18S rRNA and is generated by cleavages A$_1$ and A$_2$ (Russell & Tollervey, 1992). This pathway is infrequently used in wild-type yeast, but increased levels of the 23S rRNA have been associated with depletion of other proteins involved in pre-rRNA processing (Tollervey et al., 1991; Girard et al., 1992; Hong et al., 1997). Taken together, these results indicate that the primary defect associated with loss of Cgr1p function, either through protein truncation or depletion, is in the
formation of the 25S rRNA from the 27S pre-rRNA intermediate.

The defect in 27S pre-rRNA processing in YDA110 and YDA128 predicted that the formation of the 5′-8S rRNA should also be adversely affected (Fig. 1). We therefore examined 5′-8S rRNA synthesis in YDA110 cells by pulse labelling with [3H]uracil for 3 or 6 min followed by a chase with an excess of cold uracil for 5, 10, 30 and 60 min (for the 6 min pulse). In wild-type cells, both forms of the 5′ S RNA were evident within 5 min of chasing (Fig. 7), indicating a normal rate of processing. By comparison, the formation of the 5′-8S rRNA was delayed in YDA110 cells (Fig. 7), consistent with the defect in 27S pre-rRNA processing that was observed in this strain (Fig. 6). The processing of the 7S pre-rRNA was also delayed in YDA110 (Fig. 7), suggesting that Cgr1p may have a role in the processing of this intermediate into the mature 5′-8S rRNA. The 5S rRNA is transcribed from a separate transcription unit by RNA polymerase III (Raue et al., 1991) and although it forms a stable complex with rpL1, and is incorporated into the 60S subunit (Deshmukh et al., 1993), this experiment showed no major defects in 5S rRNA synthesis in YDA110 cells (Fig. 7).

Cgr1p contributes to nucleolar compartmentalization

Current evidence indicates that the nucleolar compartment is formed by the recruitment of processing factors by direct or indirect interactions with rDNA or the nascent RNAs (reviewed by Melese & Xue, 1995; Carmo-Fonseca et al., 2000). To determine whether Cgr1p has a role in maintaining the integrity of the nucleolar compartment, we examined the localization of the nucleolar protein Nop1p in the cgr1 truncation mutant. Nop1p is an essential nucleolar protein involved in pre-rRNA processing and ribosomal subunit assembly (Tollervey et al., 1993) and has been shown to co-localize with Cgr1p in the nucleolus (Sun et al., 2001a). In wild-type cells, the vast majority of Nop1p localized to the nucleolar region of the nucleus, with very little of the protein in the nucleoplasm (Fig. 8a). Although Nop1p was also found in the nucleolus in YDA110 cells, there was a notable increase in the amount that was present in the nucleoplasm (Fig. 8a, arrows), suggesting that the ability of Nop1p to accumulate within the nucleolus was partially impaired in this mutant.

To determine whether this impaired localization was specific to Nop1p, we examined another nucleolar...
marker, 5′-ITS1. In S. cerevisiae, the 20S rRNA is assembled as part of the 43 pre-ribosome in the nucleolus prior to export to the cytoplasm. Once in the cytoplasm the 20S rRNA is cleaved by an endonuclease to the mature 18S rRNA and the released 5′-ITS1 spacer sequence is rapidly degraded by Xrn1p (Stevens et al., 1991). The location pattern of the 5′-ITS1 RNA is an indicator of the assembly and nuclear export of the small ribosomal subunit and 5′-ITS1 can be detected by in situ hybridization with a Cy3-labelled 5′-ITS1 probe (Moy & Silver, 1999). In wild-type cells, the majority of the 5′-ITS1 RNA was found in the nucleolar crescent (Fig. 8b). By contrast, YDA110 cells showed a relative increase in the amount of 5′-ITS1 RNA in the nucleoplasm (Fig. 8b, arrows). Taken together, these results suggest that loss of Cgr1p function adversely affects the compartmentalization of these nucleolar constituents.

**DISCUSSION**

Although rDNA transcription, rRNA processing and ribosome assembly are the best characterized functions of the nucleolus, there is increasing appreciation for this organelle as a site for other cellular activities. The nucleolus has been implicated in the nuclear export of mRNAs (Kadowaki et al., 1994; Schneider et al., 1995; Tani et al., 1995), in the processing of tRNA (Bertrand et al., 1998) and in the synthesis of the RNA components for the signal recognition particle and the enzyme telomerase (Fang & Cech, 1995; Jacobson & Pederson, 1998; Grosshans et al., 2001). Furthermore, at least two yeast cell cycle regulatory proteins are sequestered in the nucleolus away from their known targets, thereby restricting functional activity to specific points in the cell cycle (San-Segundo & Roeder, 1999; Shou et al., 1999; Visentin et al., 1999). Finally, recent evidence has shown that some yeast nucleolar proteins are potent regulators of the pace of cellular senescence and in the silencing of rDNA (Gotta et al., 1997; Kennedy et al., 1997; Kaeberlein et al., 1999; Gartenberg, 2000). The nucleolus is thus a plurifunctional organelle and the proteins that localize to this compartment may have functional activities outside the nucleolus.

To investigate the possibility that the nucleolar localization of S. cerevisiae Cgr1p is due to its role in yeast ribosome synthesis, we have examined mutants deficient in Cgr1 function for abnormalities in ribosomal subunits and pre-rRNA processing. Each of the two cgr1 mutants generated for this study was deficient in the steady-state levels of 60S ribosome subunits, resulting in abnormally high levels of the 40S subunit and an increase in half-mer polysomes. Similar profiles have been described for mutants defective in components required for pre-rRNA processing or 60S subunit assembly (Ripmaster et al., 1992; Hong et al., 1997; de la Cruz et al., 1998a; Basu et al., 2001), suggesting that Cgr1p is also involved in this pathway.

Analysis of pre-rRNA processing showed that the deficit in 60S subunits in the cgr1 mutants was attributable to a primary defect in 27S pre-rRNA processing, thereby affecting the synthesis of the mature 25S and 5.8S rRNAs. The role of Cgr1p in 25S/5.8S rRNA synthesis appears to be specific because synthesis of the 18S rRNA and 5S rRNA was unaffected in either of the cgr1 mutants. This places Cgr1p in a category of proteins that influence the production of the 25S rRNA (Moritz et al., 1991; Russell & Tollery, 1992; Deshmukh et al., 1993; Berges et al., 1994; Fabian & Hopper, 1997; Hong et al., 1997; Basu et al., 2001). However, in addition to the major effects on the synthesis of the 25S/5.8S rRNAs, we also observed delayed processing of the 35S pre-rRNA and accumulation of the 7S intermediate. This suggests that Cgr1p may influence several steps in the pathway to ribosome synthesis, for which there is precedence among factors involved in pre-rRNA processing and ribosome assembly (Kressler et al., 1999), although it is also conceivable that the effect is indirect. It has been suggested that a delay in processing of 35S pre-rRNA may be a consequence of a negative feedback mechanism to slow production of the 18S rRNA whenever the formation of the 25S/5.8S rRNA is inhibited (Zanchin et al., 1997; de la Cruz et al., 1998b). However, the delay in 35S pre-rRNA processing observed in these cgr1 mutants had little effect on the overall synthesis of the 18S rRNA.

The phenotype of the cgr1 mutants resembles the phenotype described for a mutant of Dob1p, a putative ATP-dependent RNA helicase. Similar to cgr1 mutants, the dob1 mutant has a defect in the synthesis of the 5S and 25S rRNAs, a reduction in 60S subunits and an increase in 7S rRNA (de la Cruz, 1998b). The processing of the 7S rRNA involves the activity of a complex of 3′→5′ exonucleases called the exosome, and Dob1p acts as a cofactor for this complex (Allmang et al., 1999). The similarity between the dob1 and cgr1 mutants raises the possibility that Cgr1p has a direct or indirect effect on the activity of the exosome.

The yeast nucleolus occupies a crescent-shaped region of the nucleus that is approximately one-third of the total nuclear volume (Warner, 1982). The assembly of the nucleolus is thought to involve the recruitment of various processing factors through direct or indirect interactions with rDNA, nascent rRNAs, or nucleolar proteins that already reside in the nucleolus (Melese & Xue, 1995; Carmo-Fonseca, 2000). For example, Net1p preferentially binds to rDNA and is required for the recruitment of the silencing protein Sir2p into the nucleolus (Straight et al., 1999). In net1Δ cells, Nop1p redistributes over the nucleus, suggesting that Net1p is required to maintain the integrity of the nucleolar compartment. Our results indicate that cells deficient in Cgr1p are unable to retain all Nop1p and 5′-ITS1 in the nucleolar region and this would also be consistent with a role for Cgr1p in nucleolar integrity.

In contrast to highly conserved nucleolar proteins such as Nop1p (Tollervey et al., 1991, 1993), there are no clear homologues of Cgr1p in higher eukaryotes, including Drosophila melanogaster and Caenorhabditis elegans, raising the possibility that Cgr1p is either fungal
specific or highly divergent in metazoan species. If the *A. fumigatus* orthologue of Cgr1p proves to be essential for the growth of *A. fumigatus*, its apparent divergence in higher eukaryotes would make it an attractive antifungal target. Further understanding of the role for Cgr1p in fungal ribosome synthesis awaits mechanistic detail on how this protein contributes to pre-rRNA processing.

**ACKNOWLEDGEMENTS**

Supported in part by grants from the American Cancer Society (Ohio) to D. S. A., NIAID to J. C. R., NCI to T. M. and NIH to P. A. S. We thank Jay Card for assistance with photography.

**REFERENCES**


de la Cruz, J., Kressler, D., Tollervey, D. & Linder, P. (1998b). Dbl1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3′ end formation of 5′-8S rRNA in *Saccharomyces cerevisiae*. *EMBO J* 4, 1128–1140.


Received 6 September 2001; revised 22 October 2001; accepted 3 December 2001.